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Identification of proteins expressed by the root-rot fungus *Heterobasidion annosum* when growing on lignocellulose

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MicroDrivE
Microbially Derived Energy

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Identifiering av proteiner som uttrycks av Rotticka,

Heterobasidion annosum, när den växer på lignocellulosa.

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Key words: *Heterobasidion annosum,* peptide mapping, lignocellulose, ion exchange chromatography, cellobiohydrolase

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Abstract

The Root-rot fungus, *Heterobasidion annosum,* is a major pathogen on spruce and pine trees and is estimated to cause up to 20 % production loss in the forest industry in the temperate region. The genome of *H. annosum* has recently been sequenced and a total of ~280 carbohydrate-active enzymes have been predicted so far. The fungus is apparently an efficient wood degrader and may produce enzymes of interest for enzymatic saccharification of cellulose for biofuel production. The aim of this study was to see which major enzymes that are expressed and utilized. The strain that was sequenced, *H. annosum* TC-32-1, was grown in minimal medium with different lignocelluloses as carbon source. Extracellular proteins from one culture with spruce as carbon source were detected in electrophoresis after separation by cation and anion exchange chromatography. Proteins were identified by tryptic peptide mapping using MALDI-TOF mass spectrometry. We have identified 15 proteins so far which included 1 polysaccharide lyase, 10 glycoside hydrolases and 4 hypothetical proteins. Cellulase activity measurements were carried out using p-hydroxybenzoic acid hydrazide (PHBAH) as reagent for reducing sugar. One Cel7 enzyme was identified as the most expressed protein, and showed the highest cellulase activity.

Content

1 Introduction

Due to the energy crisis, people are making great effort to look for alternative energy resources. Biofuels as suitable alternatives are developing fast. Most biofuels are made from plants with high sugar or vegetable oil content, but since these kinds of plants can instead enter the human and animal food chain, producing biofuels from those plants is criticized for leading to shortage of food and increasing food prices [UN biofuels report, 2007]. Therefore, cellulosic ethanol from non-edible plant parts is attracting more intention.

Compared to producing biofuels from sugar crops or oil plants, producing lignocellulosic ethanol requires more technical problems to be solved. Before fermentation, the lignocellulose needs to be degraded to soluble monosaccharides, which means that other kinds of enzymes should be utilized to achieve this goal. To find such lignocellulases, enzymes from some fungal pathogens of trees are studied due to their wood-decaying abilities.

1.1 Lignocellulose

Lignocellulose refer to the plant biomass which is composed of cellulose, hemicelluloses and lignin [Claassen et. al., 1999]. Cellulose and hemicelluloses are tightly bound to lignin and forms a tight and resilient structure. Lignocellulose is the major component of plant cells and is the most abundant organic biomaterial in biosphere. The composition of the lignocellulose varies between different plant species. Table 1 shows the compositions of different general species.

Table 1.
Typical chemical composition of various lignocellulosic materials (Betts et al. 1991)

Raw material	Lignin (%)	Cellulose (%)	Hemicelluloses (%)
Hardwoods	18-25	45-55	24-40
Softwoods	25-35	45-50	25-35
Grasses	10-30	25-40	25-50

1.2 *Heterobasidion annosum*

Herterobasidion annosum is a common fungal pathogen that causes root rot in conifer plantations [Stenlid et. al., 2005]. It is a white-rot fungus, which can produce sheets of white, thin mycelium beneath the bark of dead/infected roots. The rot may occur in the trunk of trees without any identifiable symptom from the outside. The best growth for *H. annosum* is obtained at pH 4-4.5, 23 °C. The primary infection is carried out by basidiospores and the further infection depends on the root-root contact [Stenlid et. al., 1998].

Figure.1 *Heterobasidion annsum* near Commanster Village by J.K. Lindsey.

Economically, *Heterobasidion* is the most serious disease of conifers in the northern hemisphere and causes the largest economical loss with conifers. Damage caused by *H.annosum* includes: increased death and windthrow, decreased growth of infected trees, reduced quality of lumber, increased risk of further infection and short rotations. For example, in Sweden, the economical loss amounts to 475 million Skr per annum. [Bendz-Hellgren and Stenlid, 1995].

A genome sequencing project of *H.annosum* is running and the genome was recently published on June 1, 2009 [JGI *Heterobasidion annosum* webpage]. The haploid genome of *Heterosbasidion annosum* has been analyzed to be approximately 34 megabases with 12,000 genes models that are distributed on eleven chromosomes. 282 carbohydrate-active enzyme (CAZY) genes have been predicted in the genome, 59 are Glycosyl transferases, 179 Glycoside hydrolases, 8 carbohydrate-binding modules (CBM1, 12, 13, 21, 48), 10 EXP (Expansin-like protein), 7 Polysaccharide lyases and 19 Carbohydrate esterase. The genome information allows us to do functional and evolutionary comparisons and strengthen the research about lignin degradation, interactions with host organisms, fungal biology and evolution, etc.

1.3 Objective

Heterobasidion annosum is an efficient wood degrader that can infect and grow rapidly on trees. The long term goal of this project is to find lignocellulases from *H. annosum* with high activity that can be utilized for degradation of lignocellulose for biofuel production. To achieve this goal, it is necessary to identify proteins expressed by *H. annosum* when growing on lignocelluloses. The aim of the master project was to grow the fungus on lignocellulose as carbon source, collect the culture filtrate, fractionate the proteins by chromatography, separate proteins by gel electrophoresis and identify major proteins in the gels by peptide mapping. This will tell which enzymes that *H. annosum* is actually utilising for lignocellulose degradation. From the genome sequence information we can only figure out which repertoir of putative proteins that that the fungus migth use.

2 Materials and methods

2.1 Fungal strain and culture conditions

Heterobasidion annosum TC-32-1 was provided by Åke Olson (Swedish University of Agricultural Sciences, Sweden). It is a P-type *Heterobasidion* from North American whose genome is sequenced in the genome project [JGI *Heterobasidion annosum* webpage]. P-type indicates strains of *H.annosum* that mainly infect pine trees. The fungi were maintained in Hagem agar medium in room temperature.

Three different batches of cultivation were carried out so far in this project. Productions of extracellular proteins were from Kremer and Woods minimal medium [Kremer, S.M., Wood, P.M. 1992] [$(NH_4)_2HPO_4$ 2.6 g, 2,2-dimethylsuccinic acid 2.2 g, KH_2PO_4 1.1 g, Urea 0.6 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot H_20$ 10 mg, $CaCl_2 \cdot 2H_2O$ 74 mg, $ZnSO_4 \cdot 7H_2O$ 6 mg, $MnSO_4 \cdot 4H_2O$ 5 mg, $CoCl_2 \cdot 6H_2O$ 1 mg, Thiamin 0.1 mg in 1 L of distilled water , adjusted to pH 5.0] with different materials as carbon source.

Figure. 2: Culture 1. From the left to right is cellulose, spruce and pretreated aspen culture.

Culture 1 (the first batch, figure 2) used spruce (milled spruce heart wood), microcrystalline cellulose (Avicel) or sulphuric acid steam pretreated aspen saw dust as carbon source. The cultivation was under two-steps culture conditions to boost fungal growth at the starting phase. First, two small squares (0.5 cm X 0.5

cm) cut from Hagem agar medium with *H.annosum* TC-32-1 were used to inoculate one 0.5 L E-flask containing 200 mL Kremer and Woods medium with 1% glucose as carbon source. After incubation at room temperature for one week, 20 mL medium without any mycelium in the E-flask was poured away and the rest of the culture was stirred. About 180 mL medium containing mycelium was transferred equally to six 1 L flasks containing 150 ml Kremer and Woods medium and variable carbon source: 15 g spruce, 10 g cellulose or 10 g pretreated Aspen respectively, all were duplicated. Steady cultures shown in figure 2 were grown at room temperature and harvested after three weeks. Aspen cultures were discarded because of no growth. 400 mL Kremer and Woods minimal medium was added to each flask and after that all flasks were shaken at 80 rpm at room temperature for 2 days before filtration.

Figure. 3: Culture 2. From the left to right is cellulose, spruce and aspen culture.

Culture 2 (the second batch, figure 3) were shaken cultures which used spruce powder, cellulose (Whatman CF-11, US) or aspen saw dust as carbon source. It also followed two-steps culture conditions. First, 8 flasks of 500 mL with 100 mL Kremer and Woods minimal medium with 1% glycerol as carbon source were prepared. Two small squares (0.5 cm * 0.5 cm) cut from Hagem agar medium with *H.annosum* TC-32-1 were added to each flask. After one week incubation, three flasks of pre-culture were poured away due to contamination. Extra 10 mL glucose solution with 10 g/L was added to four pre-cultures respectively while 5 mL 1 g/10 mL glycerol was added to one pre-culture as contrast. After 1 week,

heavy inoculums in all 5 flasks were achieved. All media in five flasks were mixed together and poured equally to 6 baffled culture flasks with different culture conditions: three flasks contained 500 mL Kremer and Woods minimal medium with 20 g aspen, 20 g spruce or 20 g cellulose (Whatman CF-11) with 0.2 g arabinogalactan. Another three flasks contained 1000 mL Kremer and Woods minimal medium with 20 g aspen, 20 g spruce or 20 g cellulose (Whatman CF-11) with 0.2 g arabinogalactan. Those cultures shown in figure 3 were shaken at 80rpm at room temperature for 3 weeks before harvest.

Figure.4: Culture 3 with different carbon source. From left to right: spruce, aspen, cellulose.

Culture 3 (the third batch, static culture) also used spruce, cellulose (Whatman CF-11) or aspen saw dust as carbon source which was followed two-steps culture conditions. First, two 500 mL flasks with 100 mL Kremer and Woods minimal medium with 1 % glucose as carbon source were prepared. Two small squares (0.5 cm * 0.5 cm) cut from Hagem agar medium with *H.annosum* TC-32-1 were added to each flask. After one week incubation, mediums in two flasks were mixed together and equally poured to six 1L flasks containing 90 mL Kremer and Woods minimal medium and variable lignocelluloses: 15 g spruce, 15 g cellulose (whatman CF-11) with 0.1 g arabinogalactan or 15 g Aspen saw dust, all were duplicated. Steady cultures shown in figure 4 were grown at room temperature and harvested after four weeks. 0.5 L of 10 mM NaAc buffer pH 5 was also added to each flask and after that all flasks were shaken at 80 rpm at room temperature for 2 days before filtration.

Table 2.
Summary of cultivation series.

Cultivation series	Description
Culture 1	Static flasks, 150 mL medium with 15 g spruce, 10 g cellulose or 10 g pretreated Aspen
Culture 2	Shaken flasks, 500 mL medium with 20 g spruce, 20 g cellulose or 20 g Aspen
Culture 3	Static flasks, 90 mL medium with 15 g spruce, 15 g cellulose with 0.1 g arabinogalactan or 15 g pretreated Aspen

2.2 Protein recovery

Shaken cultures were directly filtrated while static cultures were shaken with 10 mM NaAc buffer before filtration. All cultures were filtered through glass microfiber filter (~1 um, GF-B, Whatman, US) and centrifuged at 16,000 rpm at 4 °C for 30 minutes. The lignocellulose left with mycelium was saved in bottles and kept in -20 °C freezer. The culture supernatants were filtered again via vacuum filtration through 0.2 um bottle top filter (500ml SARSTEDT, US). Filtrates were kept in 4 °C cold room.

2.3 Protein concentration determination

Protein concentrations of culture filtrates from culture 1 and culture 2 were measured by using Protein assay Dye Reagent Concentrate (Bio-Rad) with *H.jecorina* Cel7A as protein standard (CBH1 wt c3 inact (5) provided by Jerry Ståhlberg). First, 4.0 mL desalted sample from each culture filtrate was obtained by adding 3.0 mL raw culture filtrate to 10DG Columns (Econo-Pac, Bio-Rad) and eluting with 4.0 mL 10mM NaAc buffer pH=5.0. Then, a series of protein standards were prepared by diluting the protein standard, *H. jecorina* Cel7A, down to 10.14 μg/mL. After that, 800 μL of each standard and sample were transferred into clean dry Eppendorf tubes with 200 μL of dye reagent concentrate (Bio-Rad). After vortex and incubating at room temperature for 10 minutes, the absorbance of all triplicate standards and samples were measured at 595 nm in spectrophotometer (UV-1800, SHIMADZU, Japan). For filtrates from culture2, pH values were measured by pH meter (PHM210 MeterLab).

2.4 Protein separation

All four culture filtrates from culture 1 were concentrated from 750 ml to 30 ml by using Viva-Flow 50 (10,000 MWCO). Then each solution was concentrated and diafiltered three times in Viva-Spin 20 column (5'000 MWO GE healthcare) at 8,000 g, to approximately 100 μL. The time of centrifμgation varied based on different samples, from 30 minutes to 1 hour. 19 mL Finally, the samples were diluted to 5.0 mL by adding 10 mM NaAc buffer, pH 5.0. The absorbance at 280 nm of final samples was measured using Nano-drop 1000 spectrophotometer to judge how much protein that was lost in the two steps.

The conductivity of the sample of Spruce 1 culture 1 was adjusted to about 2 times the conductivity of 10 mM NaAc buffer, pH 5.0, by adding deionized water to 25.0 mL. Both cation and anion exchange chromatography were applied to separate the proteins. The cation chromatographic media was Source 30 S packed in an XK-16 column (column volume 8 mL; Pharmacia Biotech) while the anion media was Source 30 Q packed in the same type of column with same column volume. Both columns were properly cleaned and equilibrated before use. About half (12.5 ml) of the concentrated culture filtrate from the spruce 1 culture was applied on the Source 30S column, equilibrated with 10 mM NaAc pH 5.0, using an Äkta™ Explorer 10 chromatography system (GE Healthcare). 40 mL flow-throμgh fraction was collected until the absorbance at 280 nm of the outflow dropped down to 10 % of its max Abs280nm. The proteins were eluted by two salt gradients. The first started with 10 mM NaAc pH 5 buffer and continued for 100 ml (100minutes) up to 257.5 mM NaAc buffer, while the second gradient started with 257.5mM NaAc buffer and continued for 100ml (100minutes) up to 1M NaAc buffer. The first 30 fractions were saved based on their absorbance at 280 nm. The flow rate was 1 mL/minute and fraction volume was 2 mL. After the elution, the column was cleaned with 20 % EtOH. After dilution three times with 25 mM Bistris-Cl buffer, pH 6.5, the flow-through fraction collected from cation IEC was then injected on Source 30Q column, equilibrated with 25 mM Bistris-Cl buffer pH 6.5 on the same Äkta Explorer system. 40 mL flow throμgh was collected and the proteins were eluted by two salt gradients as well. The first gradient started with 25 mM Bistris-Cl buffer pH 6.5 and continued for 100 mL (100 minutes) up to 250 mM NaCl in 25 mM Bis-Tris-Cl. The later gradient started with 250 mM NaCl in 25 mM Bis-Tris-Cl and continued for 100 mL up to 1 M NaCl in 25 mM Bis-Tris-Cl. The flow rate was 1 mL/minute and fraction volume was 2 mL. All 95 fractions were saved and the column was cleaned by 20% EtOH after elution.

2.5 Cellulase activity measurements

2.5.1 Culture filtrates cellulase activity measurement

0.5 mL of each sample from culture filtrate was mixed with equal volume of cellulose (Avicel) substrate solution (Avicel 10 g/L in 0.2 M NaAc buffer pH 5.0) in 1 mL Eppendorf tube and the mixtures were incubated at 37 °C, 150 rpm for 2 hours. Samples were taken out and shaken once per half hour. After incubation, the reaction was terminated by heating at 95 °C for 10 mins. Cellulose (Avicel) was removed by vacuum filtration throμgh Glass Fiber microtiter plate filter (1.0 μm) on a vacuum filter unit. 600 uL freshly made p-Hydroxybenzoic Acid Hydrazide (PHBAH) reagent (0.1M PHBAH, 0.2M NaK tartrate, 0.5 M NaOH) was added to each 300 uL of filtrated sample in an Eppendorf tube. The mixture was boiled at 100 °C on a heating block unit for 10 minutes. Thereafter the samples were cooled on ice for 10 minutes and transferred to a quartz cuvette with a 1-cm light path. All the samples were measured at 410 nm in a

spectrophotometer (UV-1800, SHIMADZU, Japan). Samples with Accelerase™1000 (Genencor) enzyme mixture were included as positive controls and for comparison, as well as glucose standards of know concentration for creation of a calibration curve. Each measurement was performed in triplicate [Megazyme].

The assay conditions were as follows: desalted Accelerase of 0.1 μg/mL, 0.2 μg/mL, 0.5 μg/mL, and 1 μg/mL protein concentration; desalted sample from spruce I culture 1 filtrate 50.2 μg/mL; desalted sample from spruce II culture 1 filtrate 52.7 μg/mL; desalted sample from Cellulose I culture 1 filtrate 38.7 μg/mL; desalted sample from Cellulose II culture 1 filtrate 27.9 μg/mL. Glucose standards were 10 μM, 50 μM, 100 μM, 150 μM, 200 μM.

2.5.2 Cellulase activity measurement of IEC fractions

Incubation for fractions was carried out in 300 μL microtiter plate. 100 μL of sample from each fraction was added with 100 μL Avicel solution (Avicel 10 g/L, 0.02% NaN_3, 0.2 M NaAc buffer pH 5.0) in one well of the 300 μL microtiter plate. The plates were covered by silicon lids and incubated at 37 degrees, at 140 rpm for 3 hours. The plates were taken out and shaken once per half hour. The reaction was stopped by filtration through glass fiber microtiter plate filter (1.0 μm). To stop the reaction completely, 100 μL of each filtration was transferred to a well in the 1 mL microtiter plate with 200 μL freshly made PHBAH reagent as soon as possible. After heated in boiling water for 10 minutes, the plate was cooled on ice. Finally, 200 μL of sample from each mixture was transferred to a Nunc-Immuno plate (300 μL NUNC, Denmark). The samples in the plate were measured at 410 nm in an Elisa plate reader. Since each PHBAH batch varied a little, glucose standards were used to adjust the values. Glucose standards were 10 uM, 50 μM, 100 μM, 150 μM, 200 μM [Megazyme].

2.5.3 Protein concentration measurements of IEC fractions

To correlate the enzyme activity and protein concentration of each fraction, all the saved fractions were measured at 280 nm in UV-1800. Samples were transferred to quartz cuvette and poured back after measuring.

2.6 Electrophoresis

Several fractions were selected because of their values of absorbance at 280nm. To further separate proteins in each selected fraction, SDS-PAGE electrophoresis was performed on Phast gel Gradient 8-25 % (GE Healthcare) and homemade homogeneous SDS-PAGE gel.

2.6.1 Preparation of protein samples

To achieve strong and clear enough bands for peptide mapping, different methods were adopted to prepare samples from fractions.

Two methods successfully provided enough protein for gel electrophoresis.

SDS-PAGE clean up Kit (GE Healthcare) was used in the first method. The preparation was done by applying 200 µL sample from each fraction followed by the standard protocol for the Kit. Another method was by using viva-spin2 columns (10,000 and 5,000 MWCO, GE Healthcare). 300 µL of sample from each fraction was concentrated to approximately 12 µL in the viva-spin column, centrifuged at around 8000 g for 50 minutes. Thereafter the concentrated samples were mixed with 2.4 µL 5X SDS PAGE Sample Buffer, the mixture was mixed on a vortex machine and boiled in heat blocking at 95 °C for 5 minutes before loading on gels.

2.6.2 Running and developing the Gel

Electrophoresis was performed both on PhastGel Gradient 8-25 (GE Healthcare) and homogeneous SDS-PAGE gels (15% Separation gel pH 8.8, 4% Stacking gel pH 6.8). Protein samples were loaded 1 µL/lane on PhastGel and run on electrophoresis PhastSystem (Pharmacia LKB) using method for Gradient 8-25% gels. PhastGel was stained with Colloidal Blue Staining Kit (Invitrogen). Several samples were loaded 10 uL/lane on the homogeneous gel and operated in Mini-PROTEAN Tetra Cell (BIO-RAD). With SDS PAGE running buffer, gels were run with a constant current of 20 mA for about 1 hour and stained with Colloidal Blue Staining Kit (Invitrogen). Low Molecular Weight ladder (GE healthcare) was used for molecular weight determination on each gel.

Many other methods were performed to prepare the protein samples which could be found in Appendix A.

2.7 Protein identification

Identification of the proteins from selected fractions was achieved by peptide mapping which was done by Åke Engström in the Institute of medical biochemistry and microbiology in Uppsala University.

3 gels (two Phastgels and one homogeneous gel) were sent to Åke Engström. Selected bands on these gels were manually excised and digested with trypsin. The digests were analyzed by mass spectrometry. MS scans were interpreted using Mascot Server [Matrix Science] to map the proteins on a gene catalog provided by us. The gene catalog was a preliminary gene catalog extracted in March, 2009 from the *H. annosum* genome database at JGI. It contained only the top models for each gene from the automatic annotation performed at JGI. Peptide Mass Fingerprint method was adopted first, and when bands contained more than one kind of peptides MS/MS Ion Search method was used with MALDI-TOF-MS.

3 Results

3.1 Heterobasidion annosum cultivation

Prior this work, initial cultivation trials had been performed at the department. *H.annosum* TC-32-1 was cultivated in submerged culture in shaking flasks using spruce powder or cellulose (Whatman CF-11) as carbon source. However, only small amount of mycelium was obtained within 3 weeks of cultivation. It indicated that the fungus does not grow well in these conditions and/or that more dense inoculum are needed.

This study started from four bottles of culture filtrates (culture 1) which were provided by Jesper Svedberg. Since there was still lignocellulose left that proteins might bind to, the leftover lignocellulose and mycelium were saved for further elution (Jesper Svedberg, personal communication). Filtrate Culture 1-Spruce I, Culture 1-Spruce II, Culture 1-Cellulose I and Culture 1-Cellulose II were obtained. The protein concentration in these filtrates was determined by Protein assay Dye Reagent Concentrate (Bio-Rad) using *H.jecorina* Cel7A as protein standard. The concentrations and volumes of these filtrates are shown in Table.2.

For culture 2, we added glycerol instead of glucose as carbon source for the pre-cultures, because we suspected that the glucose may cause catabolite repression. As the fungus did not grow rapidly, we introduced extra glucose and glycerol to these pre-cultures. After 5 days, we got much heavier density of the mycelium and the fungus grew faster with extra glucose than with extra glycerol. The colors of these pre-cultures with extra glucose were more yellow than with extra glycerol which may be caused by metabolism. After 3 weeks cultivation, we harvested all six cultures and filtered them by glass fiber filter papers and 0.2μm bottle top filter. Myceliums were found in 5 cultures (Culture 2-Spruce I, Culture 2-Spruce II, Culture 2-Aspen I, Culture 2-Aspen II Culture 2-Cellulose I) but not in Culture 2-Cellulose II (500mL). There was more mycelium in aspen cultures than with cellulose and spruce. The color of culture cellulose II (500 mL) was yellow which might be caused by contamination. The protein concentration, pH and volume of these different culture filtrates are shown in Table 3.

For culture 3, white paper-like thin fungus can be seen at the surface of the medium. As judged visually, more fungus grew on Aspen than on spruce and cellulose. Six bottles of filtrates were collected. Residual lignocellulose and mycelium was saved after filtration. Filtrates were kept in cold room in 4℃ while others were frozen at - 20 °C.

Table.3

Protein concentration, volume, total amount of protein and pH in different culture filtrates.

Culture	Protein Concentration (ug/mL)	Volume (mL)	pH	Total protein(mg)
Culture 1-Spruce I	93	750		70
Culture 1-Spruce II	99	730		72
Culture 1-Cellulose I	72	740		53
Culture 1-Cellulose II	52	770		40
Culture 2-Spruce I	121	880	7.0	106
Culture 2-Spruce II	61	450	7.8	27
Culture 2-Aspen I	102	900	8.0	92
Culture 2-Aspen II	153	440	7.0	67
Culture 2-Cellulose I	-20*	890	7.0	0

* A negative value was obtained for Culture 2-Cellulose I, which is discussed in the discussion section.

The protein concentration of concentrated filtrates, volume and total amount of protein from Culture 1-Spruce I, Culture 1-Spruce II, Culture 1-Cellulose I and Culture 1-Cellulose II were determined as well which are shown in Table.4. Appendix B contains the raw data of Abs595 nm from protein standards and all culture filtrates.

Table.4

Protein concentration, volume and total amount of protein from different concentrated culture filtrates.

Sample	Protein Concentration (mg/mL)	Volume (mL)	Total protein (mg)
Culture 1-Spruce I	11	5	55
Culture 1-Spruce II	4	5	29
Culture 1-Cellulose I	7	5	35
Culture 1-Cellulose II	3	5	15

After concentration and buffer exchange, 79% protein was retrieved of Culture 1-Spruce I, 40% of Culture 1-Spruce II, 66% of Culture 1-Cellulose I and 38% of Culture 1-Cellulose II.

3.2 Protein fractionation and activity measurements

3.2.1 Protein fractionation

Only Culture1-Spruce I filtrate has been fractionated so far. The filtrate was first separated on a cation exchanger Source 30S column at pH 5.0. The collected flowthrough fraction was further applied on an anion exchanger Source 30Q

column at pH 6.5. The first 30 fractions eluted from the cation column were saved and were given names from C1 to C30 while all 95 fractions from the anion column were collected and named A1 to A95. Absorbances at 280 nm of all fractions were measured spectrophotometrically.

3.2.2 Cellulase activity of the culture filtrates

Results of the cellulase activity measurment of filtrate Culture 1-Spruce I, Culture 1-Spruce II, Culture 1-Cellulose I, Culture 1-Cellulose II and desalted Accelerase, using PHBAH reducing sugar assay, are shown in Table 5 . The filtrate Cellulose II has the highest cellulase enzyme activity compared to filtrates Spruce I, Spruce II and Cellulose I. But since culture Cellulose II had a yellow colour, probably due to contamination, the high activity might derive from its high background. Filtrate Spruce I, Spruce II and Cellulose I showed quite similar cellulase enzyme activity, about 60-fold lower than the activity of Accelerase.

Table.5

The amount of reducing sugar released from crystalline cellulose (Avicel) normalised to 1μg/mL protein concentration. Raw data from the experiments are provided in Appendix C.

Sample	Spruce I	Spruce II	Cellulose I	Cellulose II	Accelerase
Reducing sugar(μM)	2.7	2.5	2.7	3.7	170.5

Appendix C contains all the raw values for this result.

3.2.3 Cellulase activity of IEC fractions

The cellulase activity of the saved fractions from the ion-exchange chromatography of the culture filtrate of Culture 1-Spruce I are shown in Figure 5 and Figure 6. The cellulase activity was generally low in the cation fractions. Somewhat higher values were seen in the broad protein peak from C6 to C11, and in fractions C3 and C14, but it is difficult to judge if they reflect real cellulase activity or just sample-to-sample vaiations.

In the anion exchange chromatogram, one small protein peak/shoulder with low cellulase activity is present in A8. Then at A28, there is a large sharp protein peak with high cellulase activity. Starting from A55, one broad peak with declining activity was observed. At A68, there was a really large peak at 280 nm but with low cellulase activity. An absorbance spectrum of fraction A68 showed the highest absorbance at 220nm and does not resemble a protein spectrum. It might be mainly pigment or other compounds in A68 instead of protein. Based on their values of absorbance at 280nm, fractions C2, C6, C9, C11, C13, C18, C24, A1, A12, A15, A16, A20, A24, A28, A34, A39, A44, A49, A55, A60, A65, A68, A72 and A75 were selected for further analysis. Raw data for A280 and enzyme activity measurements of IEC fractions can be found in Appendix D.

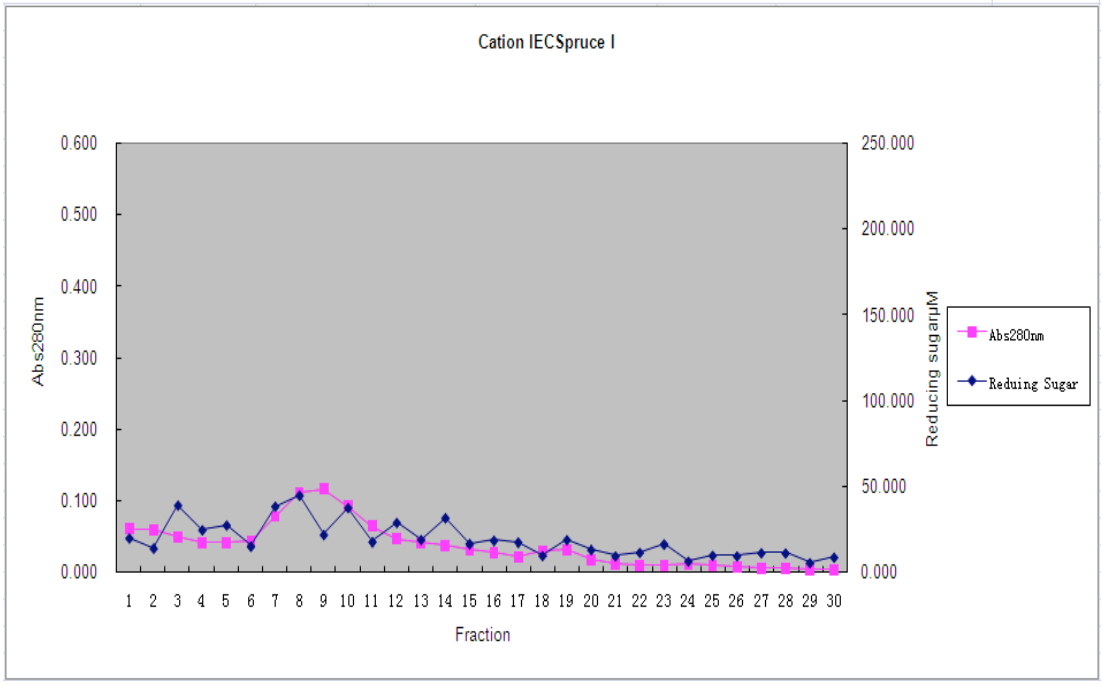


Figure.5: Abs280nm and cellulase activity of cation fractions from C1 to C30.

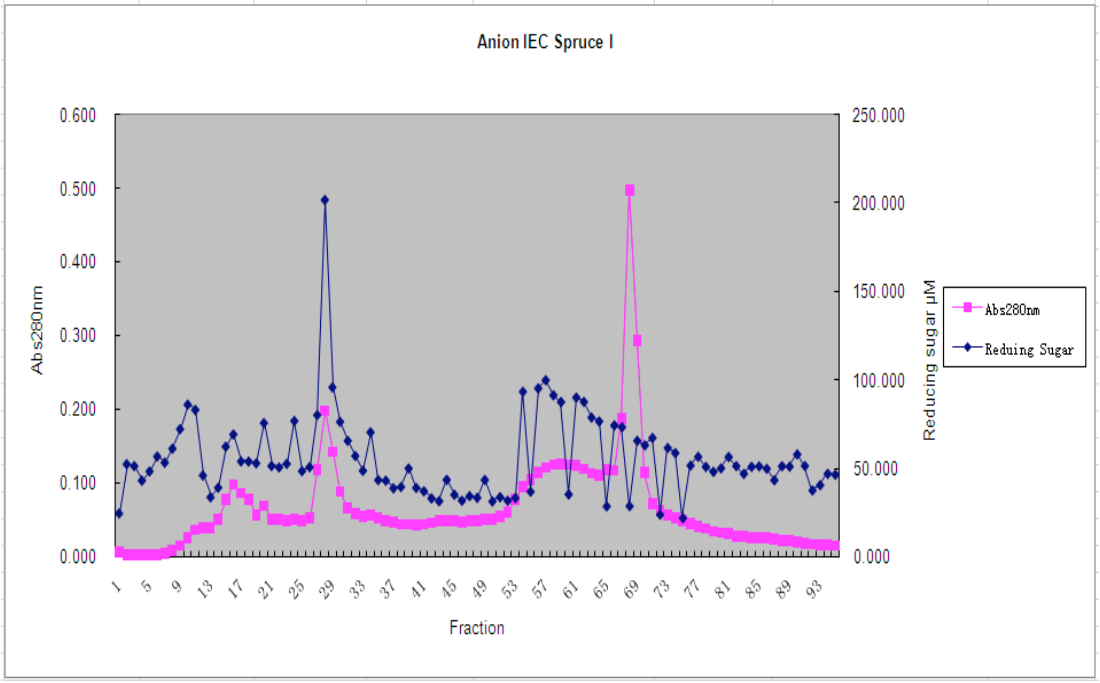


Figure.6: Abs280nm and cellulase activity of anion fractions from A1 to A95.

3.3 Gel electrophoresis

After concentration by Viva-spin2 or by SDS-PAGE Clean up kit, samples from selected fractions were loaded on different electrophoresis gels. Two Phastgel Gradient 8-25 and one homogeneous SDS-PAGE gel were successfully obtained for further peptide mapping. Figure 7, 8, and 9 show the 1DE gels that were used and 21 bands were selected for peptide mapping. Red squares indicate protein

bands for which no peptide was successfully identified. The bands with green squares indicate that at least one kind of peptide was identified and the ID above and below indicate which fraction it was and the molecular weight estimated from the protein ladder. Proteins were identified from 13 bands while there was no hit from the other 8 bands.

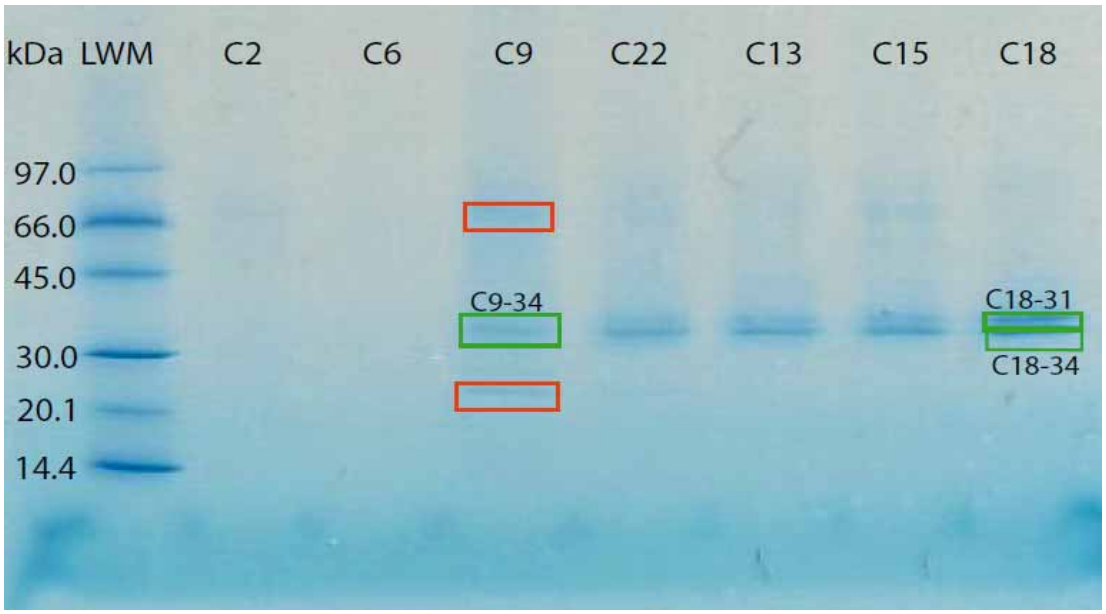


Figure 7: SDS-PAGE Phastgel Gradient 8-25 of fractions C2, C6, C9, C22, C13, C15, C18 from the cation exchange chromatography.

From the electrophoresis gel of cation fractions, 5 bands were sent for peptide mapping and the same protein was identified from C9-34, C18-31 and C18-34, a candidate pectate lyase of family PL 1 (Table 5).

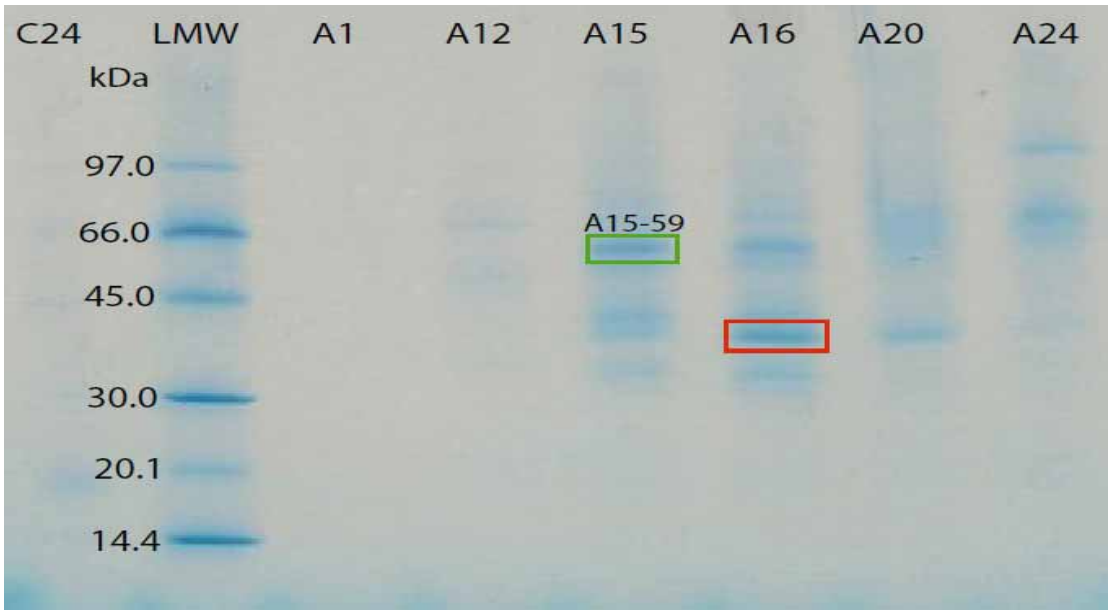
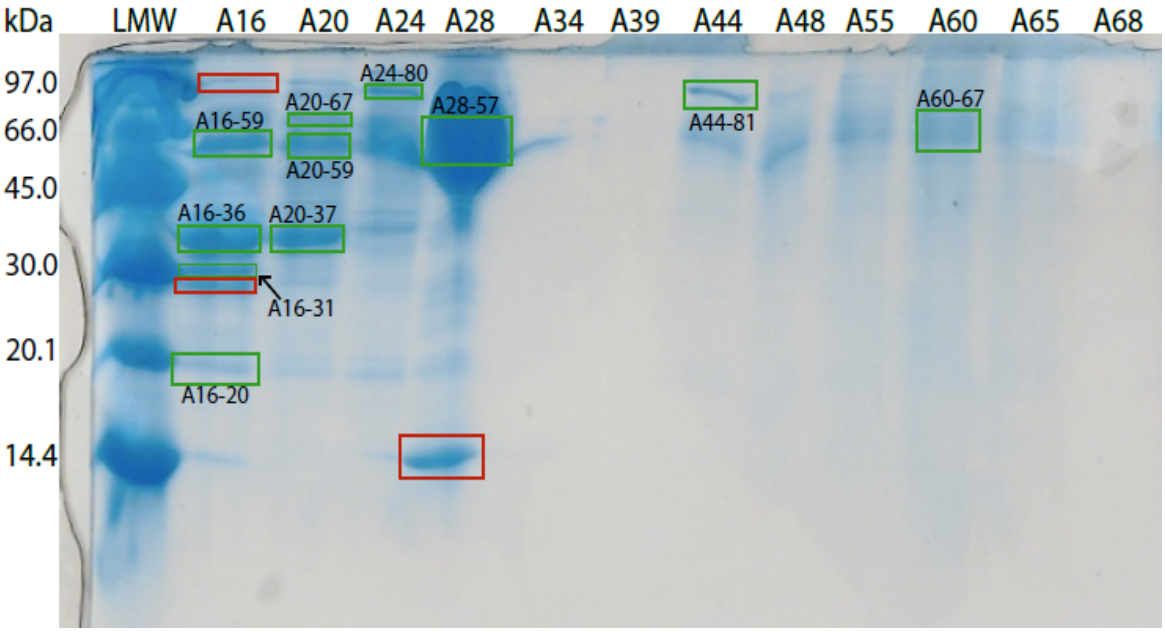


Figure.8: SDS-PAGE Phastgel Gradient 8-25 of fractions C24, A1, A12, A15, A16, A20 and A24.

Frem the gel in Figure 8, two bands were sent for peptide mapping and 1 peptide was identified from A15-59, as a candidate β-mannanase of family GH 1 (Table 5).



Figtue.9: Homogeneous SDS-PAGE gel of fractions A16, A24, A28, A34, A39, A44, A48, A55, A60and A68.

From the gel in Figure 9, 14 bands were sent for peptide mapping and 14 kinds of proteins were identified from A16-59, A16-36, A16-20, A20-67, A20-59, A20-37, A24-80, A28-57, A44-81 and A60-67, as summarised in Table 5.

3.4 Protein Identification

Bands indicated on the gels were subjected to peptide mapping by MALDI-TOF-MS after tryptic digestion. The obtained peptide masses were searched against a preliminary *H .annosum* gene catalog for identification. A total of 15 proteins were found. 13 proteins were identified by peptide mass fingerprint and 8 proteins by MS/MS Ion search. Some peptides were identified both by peptide mass fingerprint and MS/MS from different bands. The identified proteins are listed in Table 6. Details of protein identification from the bands are given in Appendix E.

Table.6

Peptide identifications matching annotated gene models in JGI *Heterobasidion annosum* database.

Model Id[a]	Description	Function	Sample[b]	Mass (kDa)[c]	Score[d]	Comment
149208	Polysaccharide Lyase Family 1 protein	candidate pectate lyase	C18-31,34* C9-34	31	107	
108918	Glycoside Hydrolase Family 3 protein	candidate α-glucosidase	A24-80	98	125	
107773	Glycoside Hydrolase Family 3 protein	candidate β-xylosidase	A24-80	81	45	
62063	Glycoside Hydrolase Family 5 protein	candidate β-mannanase	A15-59,A20-59,A16-59	44	154	N-terminal CBM1 module
66839	Glycoside Hydrolase Family 5 protein	candidate endo-β-1,4-glucanase	A20-37,A16-36	39	148	N-terminal CBM1 module
38802	Glycoside Hydrolase Family 7 protein	candidate [reducing end-acting] cellobiohydrolase	A60-67,A28-57	47	72	
36572	Glycoside Hydrolase Family 15 protein	candidate glucoamylase;	A20-67	60	53	C-terminal CBM20 module
101995	Glycoside Hydrolase Family 27 protein	candidateα-galactosidase	A20-67	49	68	N-terminal CBM1 module, Unclear signal peptide
65373	Glycoside Hydrolase Family 31 protein	candidate α-glycosidase	A24-80	79	49	Unclear signal peptide
41914	Glycoside Hydrolase Family 61 protein	candidate cell-wall active enzyme	A16-31	24	119	Unclear signal peptide and Several proteins in band
164428	Glycoside Hydrolase Family 95 protein	candidate α-1,2-L-fucosidase	A24-80	95	30	Unclear signal peptide
67586	Hypothetical Protein	Unknown	A16-20	21	55	Unidentified. Possibly a peptidase/proteinase. Matrixin-like?
165945	Hypothetical Protein	Unknown	A44-80	91	113	Similar to XP_001592187.1 among others; Intracellular? Seems to lack SP
40228	Hypothetical Protein	Unknown	A20-67	37	86	
125457	Hypothetical Protein	Unknown	A20-67	41	67	

a Gene models at http://genome.jgi-psf.org/Hetan1.

b The peptide which bands it came from.

c Molecular weight calculated from its sequence after deleting signal peptide

d Top score was showed here if the peptide was identified from different bands

4 Discussion and future perspectives

4.1 Cultivation study

As more dense inoculum was used for culture 2, we expected that there should be more protein in culture 2 filtrate than in culture 1 filtrate. But the protein concentrations of different cultures in Table 2, do not provide sufficient evidence to prove or disprove this hypothesis. Further experiments need to be carried out. The minus value of concentration of Culture 2-Cellulose I may be caused by the extremely low protein concentration and/or an inaccurate protein standard curve. As we found mycelium in Culture 2-Cellulose I, it should contain proteins in the culture. One possibility is that large amount of proteins might bind to leftover cellulose (Whatman CF-11). The saved cellulose will be washed by detergent and we will determine how much protein is bound to the cellulose. It seems that aspen is a better carbon source for *H. annosum* TC-32-1 because there was more mycelium in culture 2 with Aspen, and higher protein concentration. Also by visual observation of culture 3, it is obvious that there was more fungal growth on Aspen than spruce.

From the observation of the pre-cultures, we can conclude that glucose is a better carbon source than glycerol; although it has a potential risk to induce catabolite repression.

Based on my experience and observations during this study I recommend the following cultivation conditions for future trials: Static culture grown at room temperature, using medium adjusted to pH around 5 and with heavy inoculum.

4.2 Fractionation of the proteins

Since only a small amount of protein was retained on the cation column, it may be sufficient to use only anion IEC in the future. There was more than one kind of protein in many fractions why it may be beneficial to use a longer gradient in order to obatin better separation of proteins.

4.3 Protein identification

From the results we can definitely say that Cel7A (38802) is the major expressed protein, based on the huge dark band on the gel and the significant high peak with high cellulase activity in the chromatogram. In contrast to some other basidiomycete fungi, only one Cel7 was found in the genome. A Cel7 enzyme is often the most expressed protein in wood degrading fungi. Another white-rot fungus, *Phanerochaete chrysosporium*, has six Cel7 genes in its genome, and four Cel7 proteins were identified when it was grown on cellulose medium. GH6 family proteins are the second most expressed proteins in similar fungi. There is only one single GH6 gene in *H.annosum* genome, but we have not found it expressed in cultures so far. It might be due to several reasons: (i) the weak bands were not analyzed. (ii) The small amount of proteins in filtrates due to

cultivation conditions. (iii) The extracellular proteins might be degraded by proteases due to long cultivation time. (iv) Extracellular proteins could bind to leftover lignocelluloses.

To enhance the recovery of extracellular proteins expressed, the residual cellulose after cultivation should be washed with detergent and the released proteins extracted, quantified and identified. Other culture conditions and other strains can be tried as well. Since the strain in use is a P type *H. annosum*, one alternative is to use material from pine as carbon source. Also samples should be taken out at different time points to evaluate the influence of degradation caused by proteases.

Acknowledgements

I would like to thank my supervisors Jerry Ståhlberg and Jesper Svedberg for all their advice and help, all others in the department who help me accomplish my project.

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Appendix A

Methods performed to prepare loading samples for 1DE.

Method1: 200 μL sample from fraction was transferred to a 1 mL Eppendorf tube. Keep the lid open and put in speed vac unit to dry over night. Keep adding water to dissolve the protein in tubes until the protein was dissolved totally. Mixed with SDS PAGE loading buffer

Method2: 300μL sample from fraction was transferred to a Viva-Spin 2 5000 MWCO column. Spun down to about 20 μL and mixed with SDS PAGE loading buffer.

Method3: 100μL sample from fraction was transferred to a Viva-Spin 2 5000 MWCO column. Spun down to about 10 μL and mixed with SDS PAGE loading buffer.

Appendix B

Raw values of Abs595 nm from protein standards and all culture filtrates.

Sample ID	Concentration(μg/mL)	ABS 595nm		
For Culture1				
SP0	0.000	0.585	0.596	0.568
SP1	2.535	0.674	0.660	0.672
SP2	5.070	0.750	0.732	0.734
SP3	7.605	0.825	0.803	0.734
SP4	10.140	0.882	0.871	0.835
Spruce 1	105.243	0.746	0.713	0.740
Spruce 2	110.986	0.750	0.746	0.726
Cellulose 1	81.523	0.699	0.697	0.708
Cellulose 2	58.552	0.676	0.669	0.667
For Culture 2				
Aspen1	102	0.617	0.689	0.716
Aspen2	152	0.879	0.946	0.914
Spruce1	120	0.737	0.781	0.764
Spruce2	61	0.480	0.474	0.479
Cellulose1	-20	0.087	0.091	0.102

Appendix C

Data from enzyme activity measurements for culture filtrates

Sample	Protein Concentration(µg/ml)	Abs in 410nm
		0.870
Ha2 Gran1	50.2	0.634
		0.906
Ha2 Gran2	52.7	0.867
		0.882
		0.891
Ha2 Cellulase1	38.7	0.591
		0.704
		0.680
Ha2 Cellulase2	27.9	0.688
		0.778
		0.655
Standard Samples	**Protein Concentration(µg/ml)**	**Abs in 410nm**
Blank	0	0.323
		0.349
		0.361
S8	0.1	0.475
		0.550
		0.488
S7	0.2	0.530
		0.529
		0.625
S6	0.5	0.900
		0.965
		0.789
S5	1	1.315
		1.516
		2.119
Standard Glucose Samples	**Concentration(µM)**	**Abs in 410nm**
G1	200	1.430
		1.496
		1.391

G2	150	1.184
		1.161
		1.177
G3	100	0.783
		0.828
		0.795
G4	50	0.484
		0.498
		0.479
G5	10	0.255
		0.260
		0.271
Blank	0	0.202
		0.200
		0.187

Appendix D

Absorbance at 280nm and raw data for enzyme activity measurements of all fractions

Spruce I Cation

Fraction No.	**Abs 280nm**		**Abs 410nm**	
1	0.060	0.189	0.220	0.225
2	0.059	0.200	0.197	0.198
3	0.049	0.296	0.246	0.245
4	0.042	0.230	0.219	0.223
5	0.042	0.233	0.229	0.232
6	0.043	0.202	0.200	0.203
7	0.077	0.269	0.252	0.261
8	0.111	0.271	0.279	0.283
9	0.116	0.224	0.221	0.221
10	0.092	0.260	0.257	0.258
11	0.064	0.210	0.205	0.213
12	0.046	0.234	0.240	0.232
13	0.041	0.218	0.211	0.210
14	0.038	0.249	0.233	0.246
15	0.031	0.209	0.204	0.205
16	0.028	0.205	0.207	0.211
17	0.021	0.205	0.200	0.210
18	0.030	0.187	0.183	0.185
19	0.032	0.202	0.201	0.223

20	0.018	0.203	0.181	0.197
21	0.012	0.181	0.188	0.182
22	0.010	0.191	0.187	0.190
23	0.010	0.189	0.197	0.220
24	0.011	0.174	0.171	0.179
25	0.009	0.195	0.174	0.185
26	0.007	0.179	0.185	0.187
27	0.005	0.187	0.187	0.192
28	0.005	0.185	0.187	0.193
29	0.003	0.189	0.165	0.165
30	0.003	0.178	0.182	0.185

Spruce I Anion

Fraction No.	**Abs 280nm**		**Abs 410nm**	
1	0.006	0.232	0.231	0.231
2	0.002	0.300	0.301	0.298
3	0.002	0.281	0.312	0.297
4	0.002	0.274	0.266	0.284
5	0.002	0.295	0.286	0.285
6	0.002	0.315	0.310	0.309
7	0.004	0.307	0.299	0.300
8	0.008	0.313	0.319	0.338
9	0.015	0.358	0.357	0.344
10	0.026	0.385	0.380	0.406
11	0.036	0.406	0.387	0.354
12	0.039	0.299	0.298	0.296
13	0.039	0.248	0.275	0.264
14	0.050	0.266	0.279	0.292
15	0.078	0.342	0.346	0.356
16	0.098	0.364	0.365	0.379
17	0.086	0.335	0.325	0.315
18	0.078	0.314	0.335	0.325
19	0.056	0.304	0.317	0.344
20	0.069	0.383	0.390	0.395
21	0.051	0.314	0.320	0.317
22	0.050	0.315	0.312	0.316
23	0.049	0.326	0.325	0.311
24	0.050	0.392	0.388	0.399
25	0.049	0.308	0.306	0.310
26	0.052	0.324	0.302	0.319
27	0.118	0.414	0.408	0.396

28	0.198	0.786	0.791	0.760
29	0.142	0.441	0.467	0.455
30	0.088	0.392	0.377	0.413
31	0.066	0.361	0.362	0.360
32	0.058	0.335	0.315	0.354
33	0.054	0.320	0.304	0.302
34	0.056	0.361	0.369	0.389
35	0.052	0.292	0.299	0.286
36	0.048	0.297	0.286	0.291
37	0.047	0.284	0.289	0.261
38	0.044	0.286	0.284	0.271
39	0.044	0.315	0.304	0.311
40	0.043	0.268	0.287	0.281
41	0.044	0.283	0.269	0.266
42	0.046	0.270	0.264	0.247
43	0.048	0.252	0.263	0.252
44	0.048	0.293	0.288	0.289
45	0.048	0.292	0.253	0.255
46	0.047	0.262	0.246	0.261
47	0.048	0.257	0.268	0.268
48	0.049	0.264	0.266	0.254
49	0.050	0.291	0.288	0.291
50	0.050	0.262	0.249	0.254
51	0.054	0.262	0.266	0.259
52	0.060	0.248	0.262	0.259
53	0.078	0.262	0.269	0.252
54	0.095	0.408	0.413	0.409
55	0.105	0.270	0.261	0.277
56	0.114	0.420	0.419	0.406
57	0.121	0.420	1.933	0.435
58	0.125	0.422	0.400	0.392
59	0.126	0.393	0.387	0.403
60	0.125	0.263	0.263	0.268
61	0.124	0.395	0.413	0.395
62	0.119	0.398	0.396	0.390
63	0.113	0.397	0.371	0.344
64	0.110	0.358	0.371	0.365
65	0.118	0.239	0.245	0.247
66	0.117	0.366	0.354	0.356
67	0.187	0.359	0.356	0.353

68	0.498	0.243	0.244	0.245
69	0.294	0.345	0.343	0.318
70	0.114	0.351	0.312	0.322
71	0.072	0.340	0.345	0.334
72	0.063	0.228	0.230	0.230
73	0.056	0.320	0.328	0.325
74	0.052	0.319	0.325	0.306
75	0.048	0.221	0.223	0.225
76	0.045	0.290	0.305	0.298
77	0.041	0.321	0.311	0.301
78	0.038	0.297	0.298	0.292
79	0.034	0.295	0.294	0.275
80	0.032	0.294	0.297	0.289
81	0.031	0.325	0.311	0.296
82	0.028	0.286	0.312	0.292
83	0.027	0.290	0.281	0.284
84	0.025	0.286	0.292	0.309
85	0.025	0.301	0.297	0.291
86	0.025	0.298	0.287	0.293
87	0.023	0.290	0.263	0.273
88	0.021	0.289	0.292	0.309
89	0.021	0.287	0.296	0.305
90	0.020	0.306	0.324	0.314
91	0.018	0.314	0.298	0.279
92	0.017	0.256	0.261	0.262
93	0.016	0.263	0.267	0.273
94	0.016	0.282	0.277	0.296
95	0.015	0.286	0.278	0.287

Appendix E

Detailed information of protein identification.

ID	Culture	Fraction	Gel/Identification	Quality
S1-A28-55-01	Spruce 1	Anion 28, 60	Gel 3: f3, f1	3.20E-06
S1-A44-90-01	Spruce 1	Anion 44	Gel 3, f2	6.10E-08
S1-A24-80-01	Spruce 1	Anion 24	Gel 3, f5	3.90E-09
S1-A24-80-02	Spruce 1	Anion 24	Gel 3, f5	1.50E-01
S1-A24-80-03	Spruce 1	Anion 24	Gel 3, f5	4.10E-01
S1-A24-80-04	Spruce 1	Anion 24	Gel 3, f5	1.10E+01
S1-A20-40-01	Spruce 1	Anion 20	Gel 3, f6 (ms/ms)	1.70E-11

S1-A16-60-01	Spruce 1	Anion 16	Gel 3, f7 (ms/ms), Gel 1 f5	1.00E-15
S1-A16-32-01	Spruce 1	Anion 16	Gel 3, f8 (ms/ms)	8.90E-10
S1-A16-20-01	Spruce 1	Anion 16	Gel 3, f10 (ms/ms)	1.00E-05
S1-C18-33-01	Spruce 1	Cation 18	Gel 1, f1, f2, f4	2.40E-07
S1-A20-65-01	Spruce 1	Anion 20	Gel 3.1, f1 (ms/ms)	4.80E-05
S1-A20-65-02	Spruce 1	Anion 20	Gel 3.1, f1 (ms/ms)	4.20E-07
S1-A20-55-01	Spruce 1	Anion 20	Gel 3.1, f2 (ms/ms)	3.50E-05
S1-A20-55-02	Spruce 1	Anion 20	Gel 3.1, f2 (ms/ms)	1.80E-06

ID	Gene model	Coordinates	Family	Description
S1-A28-55-01	38802	scaffold_11:1593458-1595500	GH 7	Glycoside Hydrolase Family 7 protein
S1-A44-90-01	165945	scaffold_1:52455-55226		
S1-A24-80-01	108918	scaffold_15:886980-890661	GH 3	Glycoside Hydrolase Family 3 protein
S1-A24-80-02	65373	scaffold_8:350912-354500	GH 31	Glycoside Hydrolase Family 31 protein
S1-A24-80-03	107773	scaffold_12:1270033-1272512	GH 3	Glycoside Hydrolase Family 3 protein
S1-A24-80-04	164428	scaffold_13:368494-372262	GH 95	Glycoside Hydrolase Family 95 protein
S1-A20-40-01	66839	scaffold_11:1030791-1032782	GH 5	Glycoside Hydrolase Family 5 protein
S1-A16-60-01	62063	scaffold_3:2760993-2763350	GH 5	Glycoside Hydrolase Family 5 protein
S1-A16-32-01	41914	scaffold_1:1720429-1721353	GH 61	Glycoside Hydrolase Family 61 protein
S1-A16-20-01	67586	scaffold_13:63421-64316		
S1-C18-33-01	149208	scaffold_16:256183-257828	PL 1	Polysaccharide Lyase Family 1 protein
S1-A20-65-01	36572	scaffold_7:703865-706371	GH 15	Glycoside Hydrolase Family 15 protein
S1-A20-65-02	101995	scaffold_3:2548095-2550376	GH 27	Glycoside Hydrolase Family 27 protein
S1-A20-55-01	40228	scaffold_17:269216-271159		
S1-A20-55-02	125457	scaffold_8:612473-614165		

ID	Function	Comment	pI
S1-A28-55-01	candidate [reducing end-acting] cellobiohydrolase		4.51
S1-A44-90-01	Unidentified. Similar to XP_001592187.1 among others	Intracellular? Seems to lack SP	4.63
S1-A24-80-01	candidate b-glucosidase		5.21
S1-A24-80-02	candidate a-glycosidase	Unclear SP	4.92
S1-A24-80-03	candidate b-xylosidase		4.77
S1-A24-80-04	candidate a-1,2-L-fucosidase	Unclear SP	4.85
S1-A20-40-01	candidate endo-b-1,4-glucanase; N-terminal CBM1 module		4.43
S1-A16-60-01	candidate b-mannanase; N-terminal CBM1 module		4.66
S1-A16-32-01	candidate cell-wall active enzyme	No SP? (Several proteins in band)	4.94
S1-A16-20-01	Unidentified. Possibly a peptidase/proteinase. Matrixin-like?		4.5
S1-C18-33-01	candidate pectate lyase		6.24

S1-A20-65-01	candidate glucoamylase; C-terminal CBM20 module		4.74
S1-A20-65-02	candidate a-galactosidase; N-terminal CBM1 module	Unclear SP	4.65
S1-A20-55-01	Unidentified		4.51
S1-A20-55-02	Unidentified		4.42

ID	Mass from seq	Mass from band
S1-A28-55-01	46978.5	55000
S1-A44-90-01	91264.2	90000
S1-A24-80-01	98014.9	80000
S1-A24-80-02	79336	80000
S1-A24-80-03	80564.3	80000
S1-A24-80-04	95409	80000
S1-A20-40-01	38930.1	40000
S1-A16-60-01	44308.5	60000
S1-A16-32-01	24305.9	32000
S1-A16-20-01	20517.2	20000
S1-C18-33-01	30800.8	33000
S1-A20-65-01	59551.4	65000
S1-A20-65-02	48799.6	65000
S1-A20-55-01	37342.8	55000
S1-A20-55-02	40984.2	55000